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A STUDY OF "IMMEDIATE" SENSITIZATION BY ADSORPTION OF

ANTIGENS AND ANTIBODIES IN VITRO

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PREAMBLE

Most of the studies reported in the succeeding pages were made during the period of the principal investigator's sabbatical leave as Senior Fellow of the National Science Foundation at the Sir William Dunn School of Pathology at Oxford.

Although the principal investigator was on academic leave from Stanford he was not on leave from his research obligations, and continued to direct operations from Oxford. The directing of research by correspondence is never easy: it is doubly difficult from abroad. The success of the investigations and the volume of work done is owed to the excellent coordination of the support by the base laboratory at Stanford and for this the principal investigator is particularly grateful to Mrs. Virginia Cox and to Mrs. Nancy Peterson without whose devotion to the administrative, logistic, and scientific problems involved the project would never have been undertaken.

The principal investigator is also very grateful to the London Office of the ONR for expediting the exchange of samples between the two research stations.

George A. Feigen

FUTURE PLANS

The general aim of the studies contemplated is to continue to eluci-
date the nature of in vitro sensitization with various types of purified
and specific immunological reactants. Since the prime target in the
anaphylactic reaction is a cell which has the capacity of releasing humoral
substances upon being insulted, it is particularly valuable to work with
systems which are highly selective in their effect. Thus the systems of
interest are subject to a double restriction: they must be antigenic,
hence protein in nature, and they should be cell-specific. In the present
report we have dealt with 4 types of systems, all of which are capable of
producing anaphylaxis and 3 of which are capable of specific insult apart
from anaphylaxis. The systems under consideration are:

1. Ovalbumin-antiovalbumin : non selective
2. Streptolysin   : serotonin-releasing
3. Sea urchin toxin   : histamine-releasing
4. Tetanus (NSP)   : cholinergic

One of the most important questions in this field is what is the
immune number of molecules per cell necessary to cause injury. For iso-
lated mast cells this figure now lies in the range between 10^6 and 50
and for whole tissues the answer can be nowhere that clear. However a
figure can be arrived at if one knows that cellular release is quantal
and there are separate agents available for total release and specific
release. Total release can be secured, for example, by distilled water
or ammonia, while specific release can be obtained by the several agents
now under test.

The mechanism of sensitization with antigens and antibodies has been
studied in this laboratory during the past years with particular emphasis
on antibody fixation, and this process is now well enough understood in
operational terms to have provided a fairly general quantitative theory.
A principal objective will continue to be the detailed study of critical
conditions modifying this process. Since the antibody to be used for any
type of subsequent study has to be restricted to a species which is known
to sensitize guinea pig tissues--i.e., rabbit γ-globulin--that part of
the reaction will be known for the entire array of antigens, hence the
emphasis during the next year will be to continue with such work as will
establish the degree of quantitative toxicological specificity of the
several proteins now under investigation.

The specific goals for next year's work are outlined below.

1. Ovalbumin-antiovalbumin

   A. Continuation of present studies of modulating fixation
      reaction of antigen and antibody

      1. Anions and cations of the Hofmeister Series
      2. Urea-treated antibody
      3. Enzyme digested antibody

B. Continuation of studies of kinetics of sensitization

C. Initiation of studies on exchange desorption with non-antibody γ-globulin of the same and different species, with whole and enzyme-digested reagents.

    1. Recently sensitized tissues
    2. Chronically sensitized tissues

II. Sea Urchin Toxin

A. Continuation of present studies on the kinetics of release of humoral agents from the gut and heart.

B. Study of water and electrolyte economy associated with toxicity.

C. Physical-chemical studies of toxin.

D. Initiation of experiments to produce toxoid for production of specific reagents for immunochemical study.

III. Streptolysin

A. Completion of present experiments on electrolyte economy, hemolysis with crude material. Completion of study on known serotonin and blocking agents.

B. Studies with purified material to determine whether humoral release changes dose-response values of isolated tissues.

C. Electrophysiological studies in single atrial cells.

D. Sensitization studies.

IV. Properties of Non Spasmogenic Factor and its Corresponding Equine Antibody

(entries marked with * either completed or in progress)

I. Preparation and Purification of NSP

  * A. Protagon adsorption
  * B. Cerebroside-ganglioside adsorption
  * C. Acid precipitation
      D. Ammonium sulfate fractionation
      E. Gradient-elution chromatography

II. Characterization of Antigen

    A. Physico-chemical
*   1. Protein content
*   2. UV absorption
*   3. Ultracentrifugation
        4. Isoelectric point
        5. Stability: time-temperature

    B. Immunological
*   1. Lf titrations on fractions
*   2. Gel-diffusion and immunoelectrophoresis
        3. Sensitization

    C. Biological
*   1. Lethal toxicity
        2. MEPP (Electrophysiological)
*   a. fractions
           b. temperature dependence of fractions' effects
           c. protection by antitoxin

III. Characterization of Antibody

    A. Physico-chemical
*   1. Heterogeneity--fractionation and ultracentrifugation
        2. Viscosity
        3. Immunoelectrophoresis

    B. Immunological (with toxoid, toxin, and NSP)
        1. Seroflocculation
           a. Lf units
*   b. effect of AB concentration on optimal proportions
*   c. quantitative precipitation
           d. relation of [AB], [AG] to Lf unit

        2. Specificity
*   a. absorption of antibody with CGC complex
           b. immunodiffusion
           c. protection with absorbed antitoxin

        3. Other features
           a. complement fixation
           b. inhibition

CONTENTS:

PART I

VELOCITY OF SENSITIZATION OF NORMAL TISSUE WITH ANTIOVALBUMIN

I. VELOCITY OF SENSITIZATION OF NORMAL TISSUES WITH ANTIOVALBUMIN

Continuing work in this laboratory has shown that the process of sensitization of guinea pig gut with rabbit antiovalbumin is critically dependent on the temperature of the incubation and post-incubation phases. If the incubation temperature was low the reaction could be enhanced by increasing the post-incubation temperature; but if it was high, a drop in post-incubation temperature did not reduce the reaction of the tissue at challenge. Further information about the reaction was that the length of the incubation period affected the velocity of sensitization but not its final magnitude, that the reaction appeared to follow first order kinetics for a significant portion of its course, and that the relationship between antibody concentration and velocity could be efficiently described by the general form of the Michaelis equation. From a system of experiments in which both antibody concentration and temperature were variables it was concluded that if the pre-incubation temperature was varied but the challenge temperature kept fixed, at a constant reaction time before challenge there resulted a family of curves which reached the same maximum with antibody concentration but were more skewed as the temperature was dropped. On the assumption that the kinetic order did not change with temperature and that the reaction values were kinetic outcomes of the combined effects of concentration and temperature after a constant reaction time, it was possible to calculate an activation energy for the process of sensitization as being about 12 kcal.

These results provided a sufficient basis for a mathematical analysis of the problem from which emerged two important inferences about the process of sensitization: 1. sensitization entails a loss of entropy, hence it is a process having some similarity to adsorption; 2. the kinetics of sensitization can be made dependent on the equilibrium constant of adsorption in a two-step reaction sequence. The argument follows below.

Entropy of Activation:

The energy terms cannot be isolated by classical methods but a certain amount of information may be obtained by the application of the theory of absolute reaction rates. In the usual notation the equilibrium constant, K^*, for the formation of the activated complex is given by

$$K^* = e^{-\Delta F^*/RT} = e^{-\Delta S^*/R}\, e^{-\Delta H^*/RT} \tag{1}$$

in which these terms have the usual meaning in absolute reaction rate theory. The relationship between the specific velocity constant, k, and the equilibrium constant, K^*, is expressed by

$$k = K^*\left(\frac{\kappa T}{h}\right) \tag{2}$$

in which κ and h are, respectively the Boltzmann constant and the Planck constant. Since the usual relationships among free energy, enthalpy, and entropy apply here, at moderate temperatures and at high activation energies

$$k = (\frac{\kappa T}{h}) \, e^{-E/RT} \, e^{\Delta S*/R} \tag{3}$$

From the calculated velocity constants corresponding to the half-reaction, 1.93×10^{-4} sec^{-1}, and the activation energy, 11.9 kcal, we obtain $\Delta S* = -38.1$ EU.

A negative entropy value implies a loss of translational and rotational degrees of freedom, which is not unusual in adsorption reactions; it suggests, in this case, that the process of sensitization is not inconsistent with a mechanism involving a cell attachment of a particular kind during an intermediate step of the reaction. If a specific configuration is necessary, there may be only a few sites on the cell for a particular attachment, there may be only certain molecules in a population capable of this attachment, or both; in any case, if the reaction depends on the formation of a particular bond, this step might become rate-limiting. The average kinetics can fit a scheme.

Reaction Sequence:

$$Ab + X \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AbX \overset{k_3}{\longrightarrow} AbX* \tag{4}$$

in which Ab is the antibody concentration of effective sites, AbX the activated complex and AbX* the reacted site. Although the final reaction between AbX* and the antigen appears to involve the liberation of a short-lived enzyme, the fixation reaction itself is probably not enzymatic as the antibody itself can be dissociated from the site and because the process appears not to be affected by the usual anti-metabolites.

A simple model can be proposed on the basis of the Langmuir Theory to account, in a general way, for a limited reaction sequence.

If the overall reaction is considered as an activated adsorption and there are S sites per cell of which a fraction, θ, is occupied in the first layer we can write the equilibrium reaction

$$k_d S\theta = k_a S(1 - \theta)C , \tag{5}$$

in which C is the antibody concentration and k_a and k_d are the rate constants for adsorption and desorption, respectively. The fraction of

occupied sites will depend on the equilibrium constant. K_e , and the antibody concentration, since

$$\frac{\theta}{1 - \theta} = \frac{k_a}{k_d} C = K_e C ; \qquad (6)$$

therefore, the proportion of sites occupied at equilibrium will be

$$\theta_{equil} = \frac{K_e C}{1 + K_e C} . \qquad (7)$$

If we suppose that some cell constituent X has to adsorb antibody in a particular way to form X* so that X* will liberate histamine quantally when antigen is added, we see that the rate of the reaction

$$X \xrightarrow{k_3} X* \qquad (8)$$

can be made dependent on the number of sites already occupied by antibody, and upon the particular restriction of temperature dependence and critical fit, if k_3 is set equal to $\kappa\theta$, in which κ is the rate constant depending on these characteristics. If a definite number of antibody molecules per cell is required for the conversion of X to X* and if the total number of such cells is limited by X^*_{max}, we can write the first order expression for this reaction as

$$1 - X^*/X^*_{max} = e^{-k_3 t} \qquad (9)$$

Since the release of histamine is quantal, X^* implies H and X^*_{max} implies H_{max};

we then have from equation (9)

$$1 - H/H_{max} = e^{-k_3 t} \qquad (10)$$

Substituting y for H/H_{max} and $\kappa\theta$ for k_3

$$(1 - y) = e^{-\kappa\theta t} \qquad (11)$$

and putting in the equilibrium expression for θ

$$(1 - y) = e^{-\kappa t}\left[\frac{K_e C}{1 + K_e C}\right] \quad (12)$$

Under experimental conditions where the antibody concentration is in great excess, C can be put equal to C_0 , provided adequate time is given for equilibration if C is very low, the time necessary for equilibration may be greatly prolonged; hence the lag period absorbed at low concentrations.

As a further check on the proposition that the kinetic order does not change with temperature, it was necessary to obtain time-course data for an array of antibody concentrations of reaction occasioned by the use of heterogeneous antibody. We prepared a pool of specific antibody by dissociation of antigen-antibody complexes formed by the reaction between purified antiovalbumin (γ-globulin) and crystaline ovalbumin. These results have not yet been analyzed in detail but they show that the concentration of antibody required for sensitization can be reduced by an order of magnitude and that the reaction has the kinetic properties predicted in the preceding treatment.

Studies involving treatment of tissue surface before and after adsorption of antibody are in progress and will be reported on when they are completed.

PART II

PROPERTIES OF NON-SPASMOGENIC PRINCIPLE (NSP) OF TETANUS TOXIN
AND OF ITS CORRESPONDING EQUINE ANTIBODY

II. PROPERTIES OF NON-SPASMOGENIC PRINCIPLE (NSP) OF TETANUS TOXIN AND
OF ITS CORRESPONDING EQUINE ANTIBODY

Previous work of this group (1) has shown that there is a principle
in crude tetanus toxin which has the property of increasing the frequency
of miniature end-plate potentials (MEPP) in the isolated hemithorax of
the mouse. The ratio of the peripheral to the central (paralytic) activity
was increased by removing the greater part of the centrally acting tetano-
spasmin by specific adsorption upon protagon. The electrophysiological
properties of the impure preparation could be significantly enhanced by
temperature so as to reveal an activation energy of about 7 kcal for the
process. The effects of toxin could be blocked by appropriate doses of
curare or antitoxin. The preparation was also shown to be thermolabile.

The present studies deal with attempts to isolate this factor by
various means and are concerned with the physical-chemical properties
of the fractions in relation to their biological behavior, as estimated
by the relative potencies given by the electrophysiological and the gross
pharmacological tests.

Since most of the present work at Oxford has depended on the electro-
physiological work performed at Stanford which, by its nature, becomes
limiting, the investigations had to be carried on in "parallel" rather
than in "series" and for this reason there is not yet a complete character-
ization of each preparation. Nevertheless, it was felt that a general
outline of the plan should be presented and the extent to which the infor-
mation was complete in the various categories indicated.

A. Preparation and Purification of NSP:

1. Protagon adsorption:

The preparation of protagon and of its use as an adsorbent
for tetanospasmin has been published by van Heyningen (2) and the bio-
logical effect of the residue has been described by us (1).

2. C-G-C adsorption:

The high degree of specificity evidenced by the selective
fixation of tetanospasmin for ganglioside which was shown by van Heyningen
and Miller (3) suggested that as an elegant method of removing the para-
lytic factor, thereby enriching the preparation with respect to NSP. Two
preparations have been made according to the protocol below. These prep-
arations were made in the same way, differing only in the efficiency of
ultracentrifugation, thus the difference in potency indicates the sensi-
tivity of the biological testing with respect to slight changes in procedure.

a. Preparation of cerebroside-ganglioside complex (CGC):
A mixture consisting of 3.01 g of cerebroside and 0.9986 g of ganglioside
was dissolved in 130 ml of warm MeOH-CHCl$_3$ (1:1). The solvent was removed
under vacuum over a steam bath, and the residue was dried in vacuo for
60 minutes. The cakey white precipitate was suspended in 50 ml of boiling
water and shaken for 5 minutes. A sufficient quantity of water was then
added to bring the final volume of the suspension to 100 ml.

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b. Adsorption of tetanospasmin: Toxin preparation TD594B (Wellcome Laboratories) was prepared for adsorption by dissolving 0.9966g of the dried, lyophilized powder in 80 ml of 0.1M phosphate buffer (pH 6.98). The resulting solution was treated with 20 ml of the CGC suspension and, after vigorous shaking, aliquots were delivered into plastic cups and centrifuged at 23,500 rpm for 20 minutes in a Spinco (Model L) preparative ultra-centrifuge. The original solution was extracted 6 times in this way and all but the first two pellet residues were discarded. The elapsed time for the adsorption-centrifugation procedure was 6 days.

3. Dialysis and Lyophilization:

The supernate (110 ml) was dialyzed against three 2-liter changes of distilled water. The increment in volume after dialysis amounted to 12 ml.

4. Acid Treatment:

One half of the preparation (60 ml) was brought to pH 4. A flocculent precipitate formed overnight. The volume of the solution was then reduced to 15 ml by pervaporation and the precipitate centrifuged down. The supernate showed no material precipitable in 5% TCA. The precipitate was next dissolved by adjusting the pH of its aqueous suspension to pH 7.0 with 0.1 N NaOH. The solutions were then separately dialyzed.

5. Yield:

Solution 1 (prep 38-3) yielded 101.8 mg of solids after lyophilization. Since this represented one-half of the total batch, the percentage recovery was 0.1018/0.4983 x 100 = 20.43%. The yield of acid-precipitate from solution 2 (prep 39-4) was 15.20 mg and the percentage of recovery was 0.0152/0.4983 x 100 = 3.05%.

6. Elution of Protein from Residues:

The first two residues, 9.01 g of (37-4) and 9.73 g of (37-5), were washed 8 times with approximately 20 ml aliquots of phosphate buffer. Aliquots of each washing were precipitated with equal volumes of 10% TCA and the precipitates analyzed for nitrogen. The recoveries were 43.74 mg and 25.2 mg for (37-4) and (37-5), respectively. The cumulative recovery patterns are shown in Figure 1 and the specific rates exhibited in Figure 2.

7. Effect of Varying Centrifugation Conditions:

Preparation (16-3a) was made according to the same adsorption procedure as used in (38-3) but instead of being subjected to 6 ultra-centrifugations at 23,000 rpm it was treated as follows: the original and the second suspensions were centrifuged at 3,000 rpm while the third and fourth suspensions were centrifuged at 15,000 rpm for one hour.

The percentage yield after lyophilization was 26.75% in contrast to the 20.43% yield obtained in the case of (38-3).

B. Assay Methods:

1. Seroflocculation:

The classical method for establishing the potency of tetanus antitoxin is, of course, by the protection test in which animals are primed with various dilutions of an unknown serum and then challenged with a dose of toxin known to kill a given proportion of randomly chosen animals in a stated time. The dose of antiserum which just fails to neutralize the action of the toxin standard is called the L+ dose. For obvious reasons the potency of toxoid cannot be assessed in this way and it is therefore necessary to establish the nexus between toxin and toxoid in terms of the flocculative and protective behavior of the standardized antiserum.

Since the amounts of antigen--and, therfore, of antibody-- involved in the neutralization test are too minute to be employed in the in vitro test, seroflocculation is usually carried out with reagents in concentrations 100-to 200-fold greater than those used for bioassay

Classically, there are two techniques in immunology for establishing the potency of reactants involved in seroflocculation, and the choice between them depends on the nature of the question asked. If the potency of the antibody is unknown, the antibody concentration is held constant during the titration, and the dilution of a known antigen required to produce earliest flocculation is taken as a standard of reference; if the antigen is the unknown sought, the titer is defined by the dilution of antibody needed to give this optimum proportions point with a constant concentration of antigen.

Historically the latter method, devised by Ramon, has been used in prefernce to the form technique (the Dean and Webb titration) because usually the toxoid potency has been sought, and the L_f unit given by the Ramon titration is the so-called β-optimum. There has been con- siderable discussion (4) about the reasons why the β-optimum does not coincide with the α-optimum (as obtained in the Dean and Webb titration), but no definitive explanation has been brought forward to account for the difference.

It appears to this writer that this difference is to be expected of any system in which both the antigens and antibodies are heterogeneous because the ratio of antiserum to antigen required for optimal proportions cannot be a constant, but should vary, for example, as the concentration of antiserum is decreased and one or another of the antigenic species comes into a critical ratio with its antibody. Unfortunately, the β-optimum L_f unit was adopted as an operational point of reference in this field long before the stoichiometric behavior of antigen-antibody systems was discovered; hence, since an entire system

of international control has developed around the reliability of these tests, it will continue to be necessary to translate modern findings into the older terminology.

The aim of the present work was to isolate and to study antigens which in themselves would have none of the classical meanings, although they would be derived from parent materials whose behavior was defined in classical terms. Furthermore, the principal standard, the equine antitoxin, was to be used not only to follow changes of potency but also as a specific reagent to remove tetanospasmin from the parent toxin. It thus became necessary to study the specificity and the stoichiometry of the whole antiserum and of its separate fractions in order to select products of a high degree of specificity and avidity.

One of the first problems was to determine the relation-ship between the L_f unit and the maximal amount of antibody precipitated so as to permit a standardization of the purified antibody fractions in classical terms. According to conventional technique the seroflocculation titer determined, as mentioned earlier, with concentrated reagents is extrapolated to one unit, on the assumption that the antigen:antibody ratio providing earliest flocculation is invariant. Pilot experiments in which this proposition was tested suggested that the antibody:antigen ratio at optimal proportions varied with the antibody concentration, hence that the L_f unit could not be used as a standard of reference in itself.

The amount of antibody and antigen precipitated at the equivalence zone was studied in relation to the optimal proportions point by varying the toxoid N for an array of antibody concentrations. The results of these experiments are presented in section 4 of "Results".

2. Bioassay:

Because of the characteristic pattern of the sequelae which succeed the administration of tetanus toxin, many forms of bioassay may be used. The designation of potency may be made in terms of the kinetics of the test system or in its outcome at a constant time; in both systems it is possible to use a variety of signs for recognition ranging from minimal paralysis to death. In the present study the tetanospasmin content of the various preparations was assayed by mor-tality at 4 or at 7 days*, and the potency is expressed as LD_{50} per mg of the lyophilized prepartion.

3. Electrophysiological Tests:

Electrophysiological measurements were made on isolated intercostal preparations from 20 g male Swiss-Webster mice. Each animal

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*Conventionally, the LD_{50} is taken at 4 days but the products furnished by the Wellcome Laboratories are standardized by the 7-day assay. There is a constant relationship between the two tests which permits the trans-lation of results if the standard toxin is always tested along with the experimental fractions.

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was killed by a blow to the base of the skull; the thoracic cage was quickly presented and the entire spinal column, together with adhering musculature, was cut away in a container of well gassed (95% O_2-5% CO_2) solution described by Liley (5). One-half of the thorax was mounted in a lucite clamp and placed in a constant temperature muscle bath (6) while the second half was set aside in oxygenated medium for subsequent use. Miniature end-plate potentials were measured by means of micropipettes filled with 3M-KCl; these electrodes, which had a tip resistance of 2-12 megohms, were connected by an Ag-AgCl bridge to the input of a cathode follower circuit (6) and were displayed on a Tektronix 502 oscilloscope, and photographed by a Grass oscilloscope camera. Potentials were recorded from at least 12 cells before and after the introduction of test material. A period of 15 seconds was allowed to elapse from the time that a cell was impaled until recording was begun; after that, records of cellular activity were taken for at least 10 seconds. Potentials were considered acceptable only if the frequency remained stable and the amplitude averaged at least 250μV. The frequency rather than amplitude was considered the significant parameter.

The toxin, non-spasmogenic preparation, and purified protein solutions were all made up by dissolving the dry powder in 0.15 M phosphate buffer (pH 7.0) containing 0.1% gelatin. Aliquots of 3-5 ml of the concentrations desired for testing were delivered into 5 ml serum bottles stoppered, and stored at -20° until tested, for a period not exceeding 5 days. All materials were tested at 35.5°C, with the exception of those non-spasmogenic fractions prepared by cerebroside-ganglioside complexing, which were tested at 37.0°C. As previously reported (1) the peripheral effect of tetanus toxin upon the frequency of miniature end-plate potentials becomes more pronounced with increased temperatures, and for this reason it was decided to carry out all future electrophysiological testing at 37°C.

C. Results:

1. Physical-chemical Characteristics of Toxin and NSP:

a. Nitrogen: Total and TCA-precipitable nitrogen values were determined by means of the colorimetric Kjeldahl-Nessler micromethod customarily used in this laboratory (7). That variation of values for total N among different ampoules of preparation TD594B was greater than the error of analytical method is evident from the fact that variability of the precipitable N : total N figures appeared to be less than that found for the total N/dry weight figures. Since it is unlikely that the variation of the latter figures could have resulted from regional differences in the toxin solution introduced into the ampoules for lyophilization, the probable cause of the variation was that introduced by the necessity of having to weigh out small quantities of hygroscopic material. When greater quantities of preparation became available, permitting the use of larger initial samples, the values became more consistent. Our determinations were made in triplicate, and the mean values of the several determinations for total and TCA-precipitable nitrogen are given in Table I and presented in a derived form (per mg of dry weight) in Table II.

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The general effect of CGC treatment was to increase the total nitrogen content of the preparation without greatly affecting the amount of precipitable nitrogen present. Taking for comparison the two cases studied in greatest detail to date--TD594B and its daughter NSP 38-3, we see that whereas the protein content increased by 8.7%--from 0.43 to 0.47 mg/mg dry weight--owing to treatment, the total nitrogen per mg increased by 43.5%. Since the purpose of CGC complexing was to remove selectively one of the proteins of the mixture, the observed change in composition is *prima facie* evidence that the aim had been generally accomplished.

b. *Light absorption*: Preliminary tests of conformance to Beer's Law were made at 260, 278, and 280 mμ and the results are shown in Table III. For practical considerations absorption at 278 mμ and the relation of optical density to concentration in 0.1 M phosphate buffer (ph 7.0) will be the standard of comparison in the suceeding discussion.

The calibration plots in Figure 3 relating total N to optical density for two different NSP preparations and for two separate lots of TD594B form distinct lines, supporting the inference that a qualitative change had resulted from treatment, and indicate that the remaining material is composed of molecules having greater absorbance at 278 mμ than the original mixture. Plots of absorbance against dry weight and against protein concentration (Figure 4) exaggerated this difference. On a dry weight basis (Figure 4a) the calculated absorption values for solutions containing 1 mg/ml of solute were 1.04, 1.80, and 2.06, respectively, for the parent toxin, 39-4, and 38-3. On the basis of protein concentration (Figure 4b) absorptions for a 0.10% solution (as protein) were 2.43, 3.52, and 4.42 for the series given above. Since the NSP solutions were exhaustively dialyzed, the increased absorption could not have been due to a change in composition such as to increase the ratio of free amino acids or small polypeptide in the sample, but must have resulted from the relative enrichment of a non-adsorbable protein species. This inference is supported by calculations showing that, whereas the optical density per mg ml^{-1} of N is only 37% higher in NSP than in the crude toxin, the increase is 82% per unit concentration as protein and 98% per unit concentration as the whole material.

2. <u>Physical-chemical Characteristics of Pepsin-Digested Equine Antitoxin</u>:

Equine antitoxin FP2367 containing 2700 L$_f$ units/ml was obtained by gift from the Wellcome Research Laboratories (Beckenham, Kent) through the generosity of Dr. J. F. Fulthorpe and Miss Mollie Barr. Estimation of TCA-precipitable nitrogen gave a protein content of 5.7% for the antitoxin:

a. <u>Fractionation of Antitoxin</u>: 25 ml of antitoxin was diluted with an equal part of 1% NaCl solution and treated with one-half the volume of saturated ammonium sulfate (SAS). The suspension was adjusted to pH 7.8 with 5 <u>N</u> NaOH and placed in the cold. The precipitate which

formed in the presence of 0.33 SAS was separated by centrifugation at 0°C on the following day, dissolved in 25 ml of saline and dialyzed against several changes of borate buffer at pH 7.8. The supernate was treated with a sufficient quantity of SAS to bring the salt concentration to 0.36 saturation. These maneuvers were repeated until an array of fractions ranging from 0.33 to 0.60 SAS had been prepared. The distribution of protein according to insolubility in ammonium sulfate is presented in Table IV and is illustrated by the histogram given in Figure 5.

The total amount of protein recovered by precipitation with ammonium sulfate in the range 0.33--0.60 saturation was 719 mg or 50.46% of the protein present in the original sample. It is evident from Table IV that 75% of the protein brought down under the present conditons is precipitated between the limits of 0.33 and 0.46 SAS and that the greatest amount, 32.9% is obtained in the fraction lying between 0.33 and 0.38 SAS.

b. **Ultracentrifugation**: The sedimentation behavior of the parent antitoxin and that of the fractions was studied with the aid of an analytical ultracentrifuge (Model E--Spinco) equipped with a double sector cell. Results of ultracentrifugation at 201,366 x g are shown in Table IV.

3. **Immunochemical Characterization of Toxin, NSP, and Toxoid**:

Preliminary experiments were made to determine the changes in antigenic composition in NSP which might have succeeded upon treatment of the crude toxin with cerebroside-ganglioside complex. At this writing only the first NSP preparation of the current series (16-3a) has been tested by the modified Ouchterlony technique and by the immunoelectrophoretic method of Grabar and Williams. Typical results of these tests are shown diagrammatically in Figure 6.

a. **Microdiffusion**: High concentrations of reagents had to be employed in the micr.diffusion studies in order that definitive results be yielded in a relatively short time.

i. Simultaneous comparison of toxin, toxoid, and NSP-- Figure 6-I shows the antigenic distribution of TD594B, toxoid, and NSP 16-1a at a concentration of 5 mg/ml. Essentially toxin-antitoxin is a 5-line system while NSP and antitoxin constitute a 4-line system. Toxoid shares a minor line with both toxin and NSP but not the rest of the three bands that are common to NSP and toxin.

ii. Corroborative evidence for antigenic difference between toxin and NSP--Toxin and NSP were separately titrated with various concentrations of antitoxin to show clearly the loss of an antigenic species owing to CGC treatment. The difference between Figures 6-II and 6-III is unequivocal. The former shows toxin is a 5-line system against 15 and 30 mg/ml of TD594B and the latter shows only 4 lines against these concentrations of antibody.

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3. Immunoelectrophoresis: Immunoelectrophoresis was carried out in 0.05 μ M barbital buffer agar (pH 8.2) at 25 ma for 3 hours. Figures 6-IV, 6-V, and 6-VI compare toxin to toxoid; toxin to NSP; and NSP to toxoid. Figure 6-V shows no significant change in the electrophoretic properties between toxin and NSP. The principal difference between toxin and NSP, on the one hand, and toxoid, on the other, is that toxoid lacks an antigen migrating to the left of the solution well.

4. Variation of Optimal Proportions Point with Antibody Concentrations:

For reasons mentioned in the introduction to the present section Dean and Webb titrations were made by varying the toxoid concentration from 13 to 191 L_f u/ml for 8 concentrations of antitoxin, ranging from 27 to 235 units/ml. The flocculation time of each tube was determined in every set and at the conclusion of the entire array, all of the precipitates were analyzed and the total N precipitated was corrected for the antigen N added at the equivalence. The results obtained show that the relation between antigen and antibody required to produce earliest flocculation did not increase in a simple proportion but tended to vary sigmoidally with antibody concentration, becoming independent of the latter when the antitoxin concentration attained a value of 200-225 L_fu. These results are shown in Figure 7.

The composition of specific precipitates which brought down the maximum amount of antibody in each array tended to increase in antibody content with antibody concentration until a limiting value of 1.8 Ab N : 1 AG N was reached, as displayed in Figure 8.

These results have no doubt as to the heterogeneity of the system as suggested earlier by the immunodiffusion experiments and by the antibody fractionation studies.

5. Bioassay:

Because of the high specificity of tetanus toxin for the central nervous system, couple with the fact that its killing power is reckoned in almost molecular amounts, the assay behavior of this material is highly reproducible and, within wide limits of variation, does not depend critically on the body weight. For this reason, many laboratories have adopted variations of the bioassay so as to reduce the number of animals required for the test and to shorten the time of its performance. In a recent publication van Heyningen (8) has concluded that the LD50 can be estimated adequately by testing 2-fold dilutions of toxin in a system involving only 2 animals per dilution.

As in the present investigation it was necessary to assay materials which eventually might have a lower content of tetanospasmin it was considered imperative, at the outset, to test the correspondence among assays differing in the number of animals used and in the time required for the end point.

Preparations TD594B, 16-3a, and 38-3 were assayed by intramuscular injection as follows: TD594B was tested with 2 animals per dilution at 4 days and with 5 animals per dilution at 4 and 7 days. Preparation 16-3a was tested with 2 animals per dose for 4 days, while preparation 38-3 was assayed with 5 animals per dose at 4 and 7 days.

      a. _Effect of Duration:_ An effective comparison of the relationship between the 7-day and the 4-day end point can be made by considering the results obtained on the 5-animal test for preparations TD594B and 38-3. By reference to the appropriate curve in Figure 9 the 4- and 7-day LD_{50} values are 2.3 and 4.5×10^{-7} mg for TD594B; similarly they are 3.0 and 7.0×10^{-4} mg for 38-3. The percentage reduction in dose between the 4-day and the 7-day tests is 49% in the case of the TD594B and 57% in the case of 38-3. Whether or not the difference between the two figures is signficant remains to be established by further work.

      b. _Effect of Sample Size:_ The difference between the 2-animal and the 5-animal tests for a constant test-duration can be assessed by comparing the two assays carried out on TD594B for 4 days. The 5-animal assay gives an LD_{50} of 4.5×10^{-7} mg while the 2-animal test gives 6.1×10^{-7} mg, a difference of 36 per cent.

      c. _Effect of Treatment:_ Table V displays the LD_{50}'s obtained by several methods for the standard toxin and the various fractions. A measure of the effectiveness of purification of NSP may be got from the quotient of LD_{50}/mg of standard toxin and of the fraction of interest. The toxin/NSP quotients calculated for preparation 38-3 from the 5-animal test were 1552 and 1306 for the 4- and 7-day end points, respectively; the ratio obtained for 16-3a in the 2-animal test at 4 days was 171. Even if a 3-fold increase in toxicity is allowed for by projecting these results to a 5-animal 7-day assay the ratio would be about 152. From these results it is evident that the preparation 38-3 is significantly less toxic than 16-3a.

6. Electrophysiological Experiments:

      a. _Controls:_ Previous control studies in this laboratory had shown that neither acid-treated-boiled toxin nor neutralized toxin had electrophysiological activity. Although the reduction in heterogeneity of the function relating the concentration of material to the effect had been greatly reduced by chemical treatment and the electrophysiological activity found to be concentrated in the residue after the removal of tetanospasmin, it had yet to be shown that the activity was restricted to toxin preparations exclusively and not one attributable to proteins, generally. For this reason two purified proteins, bovine γ-globulin and bovine serum albumin, were tested by the technique described in section B-3 in concentrations somewhat higher than those used for toxin. As is shown in Tables VI and VII neither bovine γ-globulin (in concentrations ranging from 10^{-8} to 10^{-2} mg/ml) nor bovine serum albumin (in concentrations lying between 10^{-5} and 10^{-1} mg/ml) succeeded in raising the frequency of MEPPs at 35.5°C.

-13-

b. Non-spasmogenic Fraction: NSP fraction 16-3a prepared by CGC-complexing, but with minimal centrifugation, gave a positive electrophysiological result as shown by Table VIII and Figure 10. Since 16-3a is the only preparation of the current series which has been assayed for MEPP potency it is difficult, at this point, to assess the effect of the new method of preparation. However, since homogeneity is one of the criteria of purification, the efficiency of the present and the past methods of chemical treatment may be compared. By reference to Figure 10 it can be seen at once that preparation 16-3a, although less heterogeneous than the parent material, is more heterogeneous than the NSP preparation made by protagon adsorption last year. Since protagon is a less efficient adsorbent than CGC, the heterogeneity must have resulted from the less effective centrifugation of 16-3a and illustrates the sensitivity of the assay method to slight alterations in procedure. Further analysis of the signficance of the difference in the MEPP$_{50}$ positions evident between 16-3a, and the older preparation must await the assay results now being obtained on the new parent toxin.

D. Discussion:

Although the non-spasmogenic factor in tetanus toxin has not yet been obtained in sufficiently pure form for adequate characterization to be made, the weight of evidence, obtained in the past (1) and present studies, suggests that the electrophysiological properties and the paralytic effects are due to separate components of tetanus toxin and are not the result of the inactivation of one property of a single molecular species which originally had possesed both. It is also highly probable that this component is a protein.

Evidence in support of this position is based on the previous findings that NSP is non-dialyzable, heat-labile, and antigenic; and on the current results indicating the loss of an antigenic component, with concomitant reduction in paralytic toxicity. The physical characteristics of the factor exhibiting the higher MEPP$_{50}$/LD$_{50}$ ratio are quite different from those of the parent material in that the total nitrogen content is now significantly higher than that originally seen (accounting for the drop in the P/T ratio) and the light absorption at 278 mμ is approximately doubled.

Although we are not yet able to show a uniform progression of changes in physical characteristics with the toxicity indices, it is expected that the electrophysiological assay--although slow and laborious-- is sensitive enough to detect changes in experimental maneuvers. A case in point is the difference in toxicity found to exist between the first NSP preparation (1) and NSP 16-3a which we prepared from the same lot of parent material but with some modifications in procedure. Figure 10 shows that 16-3a is a poorer preparation than the first product both from the standpoint of homogeneity and from the dose required to produce a 50% increase in the electrophysiological effect.

The toxin-antitoxin system is highly heterogeneous as shown by the sero-flocculation and the quantitative precipitin tests. From the immunodiffusion results, pointing to the existence of at least 5 antigens in the parent material, and from the antitoxin fractionation experiments, indicating the presence of an array of molecular species according to solubility in ammonium sulfate solutions, it appears necessary to carry on exhaustive fractionation of the reagents in order to relate flocculative to electrophysiological behavior.

E. Summary:

Purification of crude tetanus toxin, with a view to isolating the non-spasmogenic principle, has been carried out by removing tetanospasmin by adsorption on a cerebroside-ganglioside complex.

The original material consists of at least five antigens. One of the antigens has been removed by adsorption, and since the paralytic activity has been decreased concomitantly, it is evident that the lethal antigen has been extracted by the treatment. The result of this separation is to leave a residue which is significantly different in its physical properties from those of the parent material: specifically, the total nitrogen content of the preparation is increased, the ratio of the precipitable to the total nitrogen is reduced, and the absorbance per unit dry weight and per unit protein content in solution is enhanced at 278 $m\mu$.

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TABLE I

DETERMINATIONS OF TOTAL AND PRECIPITABLE NITROGEN IN TOXIN AND NSP

Prep. 1 mg	Total N (mg)	Pptable N (mg)	P/T (%)	Protein (mg)
TD594B	0.07979	0.0689	86.3	0.4306
	0.07234	0.05297	73.2	0.3311
	(.06209)	0.06386		0.3991
	0.09121	0.07321	80.27	0.4576
	ratio only		86.32	
	0.0823			
	0.0902	0.06877	76.27	
	0.09981	0.07586	76.0	0.4741[*]
		0.07642	76.57	0.4776[*]
	0.09938			
	0.09158			
Mean	0.08541	0.06857	80.28	0.4384
16-3a	0.12925	0.10363	80.18	0.6477
38-3	0.1226	0.0717	58.48	0.4481
	0.1226	0.0774	63.13	0.4838
Mean	0.1226	0.0746	60.81	0.4659
39-4	0.1125	0.0816		
	0.1011		72 5	0.5100

* Precipitated with different lots of TCA from same pool of "total" sample.

TABLE II

PHYSICAL-CHEMICAL AND BIOLOGICAL STANDARDS OF TOXIN AND NSP

Prep. (1 mg)	mg N total	mg N pptable	P/T (%)	mg protein	OD_{278}	OD prot	No. mice	LD_{50}/mg 4 days	LD_{50}/mg 7 days	LD_{50} mg protein
TD594B	0.08541	0.06857	79.28	0.4286	1.040	2.43	2	1.64×10^6		3.826×10^6
							5	2.22×10^6		5.180×10^6
							5		4.35×10^6	1.015×10^7
16-3a Stan	0.12925	0.10363	80.18	0.6477			2	9.61×10^3		1.484×10^4
38-3	0.1226	0.0746	60.81	0.4659	2.060	4.42	5	1.43×10^3		3.069×10^3
							5		3.33×10^3	7.147×10^3
39-4	0.1068	0.0816	72.53	0.5100	1.795	3.52				

TABLE III

OPTICAL DENSITIES OF TOXIN AND NSP
PREPARATIONS AT SEVERAL WAVELENGTHS

| Preparation | CONCENTRATION | | OPTICAL DENSITY | | |
	Dry weight whole prep. mg	Total N mg	OD_{278}	OD_{280}	OD_{260}
Toxin 1. TD594B	0.9813	0.09794	1.33		
	0.4907	0.04898	0.580		
	0.2454	0.02449	0.295		
	0.1227	0.01225	0.153		
	0.0614	0.00613	0.078		
	0.0307	0.00306	0.042		
2.	0.5163	0.04388	0.524	0.493	0.655
	0.2581	0.02194	0.271	0.251	0.336
	0.1291	0.01097	0.137	0.128	0.173
	0.0646	0.00549	0.072	0.069	0.092
	0.0323	0.00275	0.039	0.037	0.050
NSP 38-3	0.530	0.0650	1.01		
	0.265	0.0325	0.525		
	0.133	0.0163	0.274		
	0.067	0.00821	0.151		
	0.034	0.00417	0.085		
NSP 39-4	0.780	0.0645	1.469	1.362	2.22
	0.390	0.0322	0.755	0.722	1.04
	0.195	0.0161	0.368	0.347	0.525
	0.0975	0.00810	0.181	0.166	0.254
	0.0488	0.00405	0.091	0.084	0.132

TABLE IV

WEIGHT DISTRIBUTION OF ANTITOXIN FRACTIONS

Sample	Saturation with $(NH_4)_2SO_4$	Total Protein Recovered (mg)	% of Protein Yield	S value (Stanford)	
32-A	0 - 0.33	54.39	7.56	4.82	
32-B	0.33 - 0.38	236.3	32.86	4.76	
32-C	0.38 - 0.424	162.6	22.61	4.66	12.35
32-D	0.42 - 0.458	136.9	19.41	4.89	10.44
32-F	0.46 - 0.511	49.50	6.88	5.15	
32-G acid	0.51 - 0.600	42.0	5.84	2.98	5.13
32-G alkaline	0.60	37.5	5.21	5.39	

TABLE V

LETHAL TOXICITIES OF PREPARATIONS BY VARIOUS METHODS OF BIOASSAY

Preparation	Parameter	2-animal 4-day	Toxin NSP	5-animal 4-day	Toxin NSP	5-animal 7-day	Toxin NSP
TD594B	LD_{50}(mg)	6.10×10^{-7}		4.5×10^{-7}		2.3×10^{-7}	
	LD_{50}/mg	1.64×10^{6}		2.22×10^{6}		4.35×10^{6}	
16-3a	LD_{50}(mg)	1.04×10^{-4}					
		9.61×10^{3}	171				
38-3	LD_{50}(mg)			7.0×10^{-4}		3.0×10^{-4}	
	LD_{50}/mg			1.43×10^{3}	1552	3.33×10^{3}	1306

TABLE VI

EFFECT OF PURIFIED PROTEIN (BGG) ON FREQUENCY OF MEPPS (35.5°C)

Bath Concentration mg/ml (64 ml bath)	Control Freq.	Test Freq.	Change %
1.132×10^{-2}	5.46 ± 0.74	4.91 ± 0.90	-10.1
1.132×10^{-4}	5.15 ± 1.07	5.27 ± 1.61	+ 2.3
1.132×10^{-6}	4.26 ± 1.00	4.02 ± 0.97	- 5.6
1.132×10^{-8}	3.41 ± 0.45	3.26 ± 0.50	- 4.4

n = 6 for all concentrations

BGG Solution 0.7256 gm BGG into 100 ml buffer = 7.256×10^{0} mg/ml

TABLE VII

EFFECT OF BSA ON FREQUENCY OF MEPPS (35.5°C)

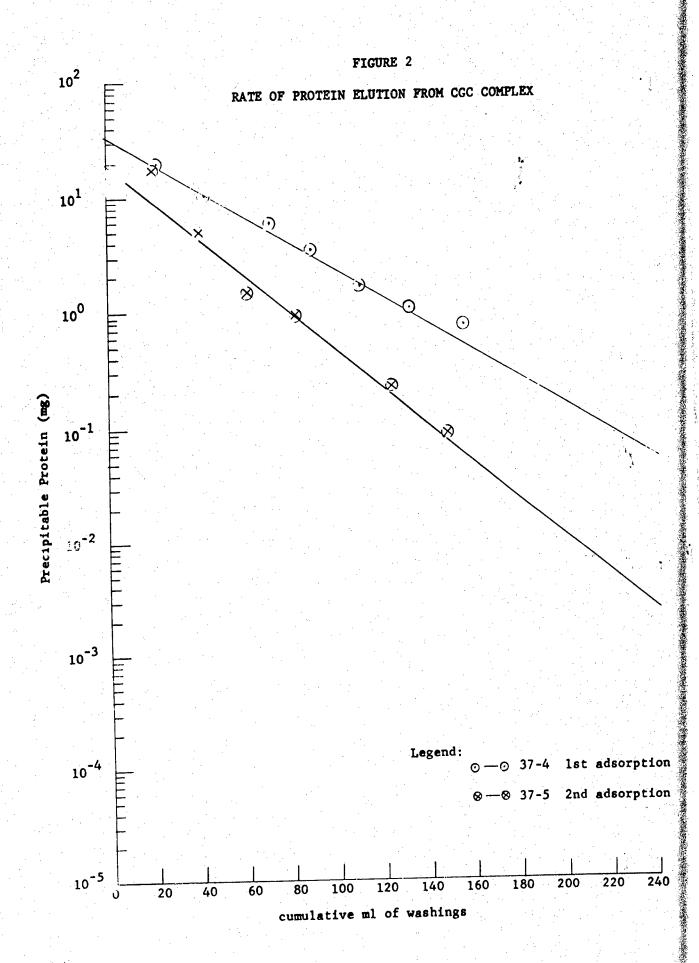| Bath Concentration mg/ml	Control Freq.	Test Freq.	Change %
1.134×10^{-1}	3.94 ± 0.47	3.72 ± 0.53	- 5.6
1.134×10^{-3}	3.81 ± 0.58	3.77 ± 0.74	- 1.0
1.134×10^{-5}	5.58 ± 0.66	5.67 ± 0.90	+ 1.6

n = 6 for all concentrations

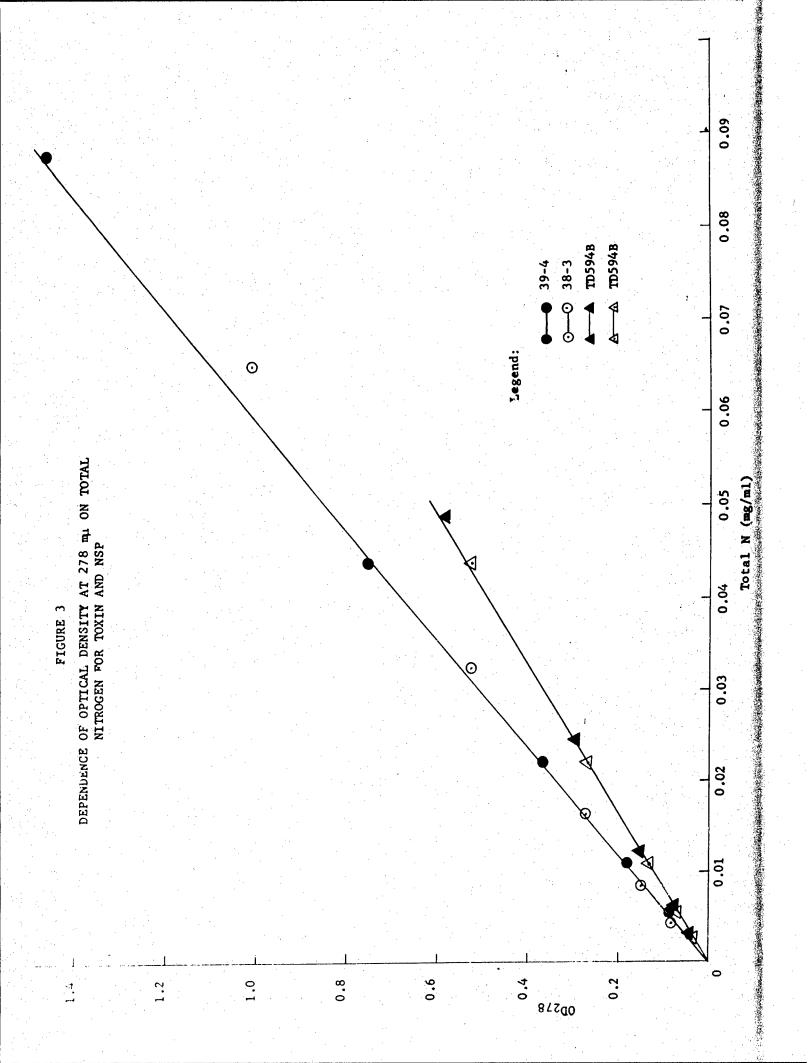BSA Solution 0.7256 gm BSA into 100 ml buffer = 7.256×10^{0} mg/ml
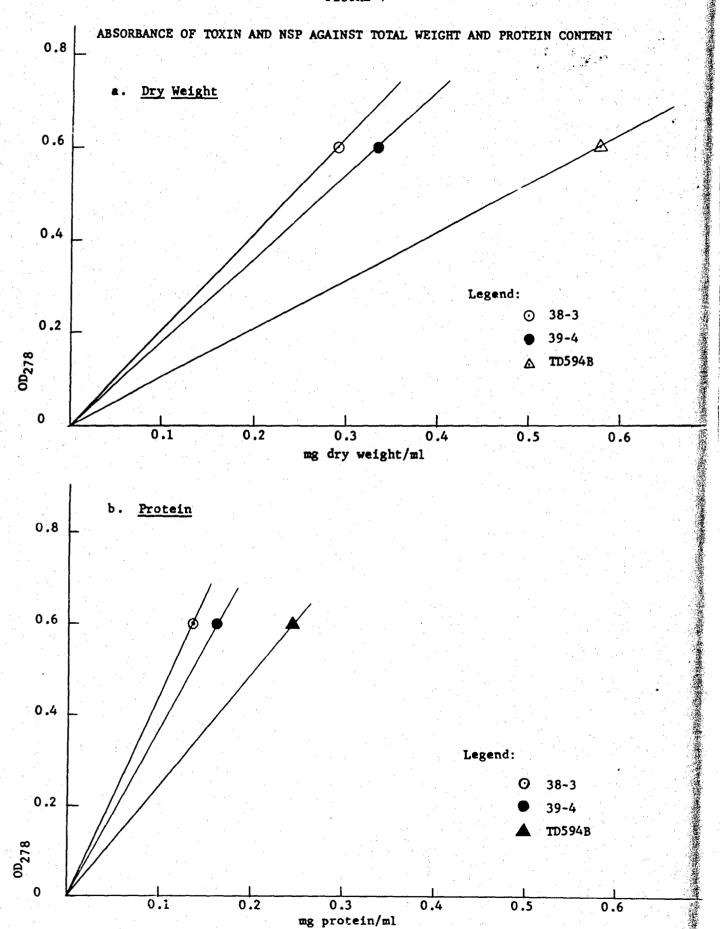
TABLE VIII
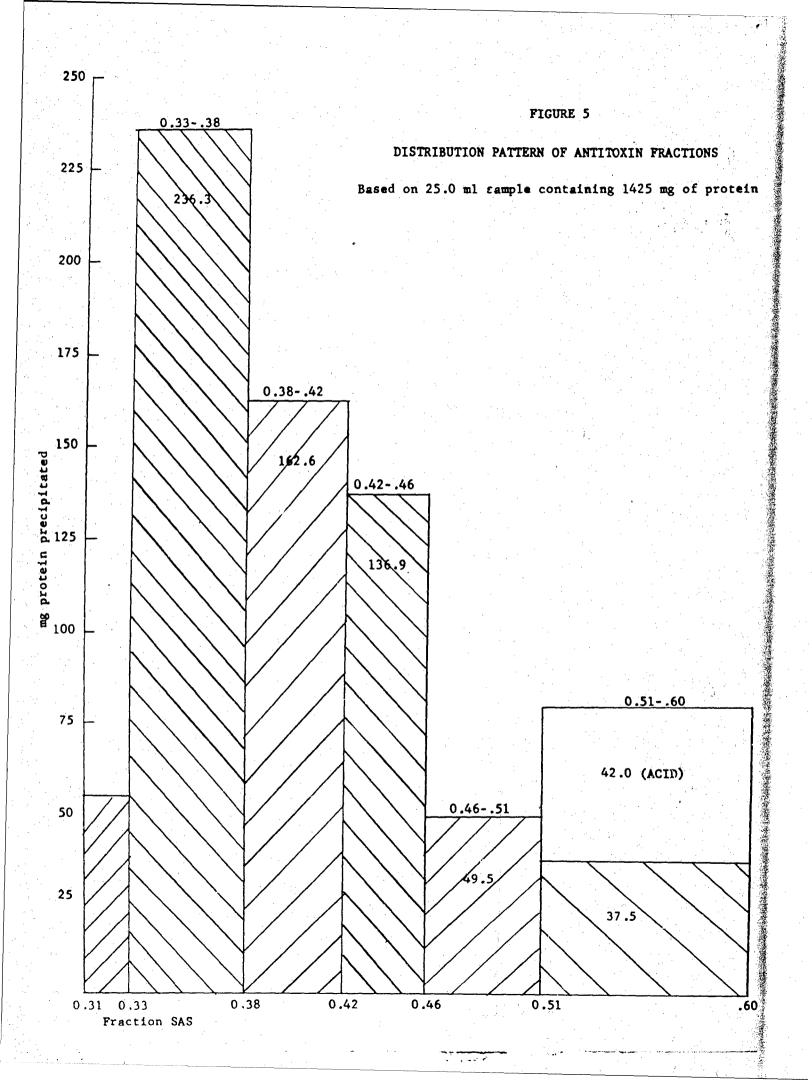
EFFECT OF MSP 16-3a ON FREQUENCY OF MEPPS (37.0°C)

Bath Concentration Final mg/ml	Control Freq.	Test Freq.	Change %	n
5.58×10^{-7}	6.57 ± 0.57	9.23 ± 1.04	$+40.5$	3
5.58×10^{-8}	8.13 ± 1.72	9.97 ± 0.59	$+21.4$	4
5.58×10^{-9}	12.08 ± 1.49	12.10 ± 2.25	$+1.6$	4

FIGURE 1

CUMULATIVE RECOVERY OF PROTEIN BY ELUTION OF CGC COMPLEX

Legend:
●—● 37-4
X—X 37-5

cumulative mg protein recovered

Total ml of PO$_4$ wash

FIGURE 2

RATE OF PROTEIN ELUTION FROM CGC COMPLEX

Legend:

⊙ — ⊙   37-4   1st adsorption

⊗ — ⊗   37-5   2nd adsorption

Precipitable Protein (mg)

cumulative ml of washings

FIGURE 3

DEPENDENCE OF OPTICAL DENSITY AT 278 mμ ON TOTAL
NITROGEN FOR TOXIN AND NSP

Total N (mg/ml)

OD278

Legend:

● 39-4
⊙ 38-3
▲ TD594B
△ TD594B

FIGURE 4

ABSORBANCE OF TOXIN AND NSP AGAINST TOTAL WEIGHT AND PROTEIN CONTENT

FIGURE 5

DISTRIBUTION PATTERN OF ANTITOXIN FRACTIONS

Based on 25.0 ml sample containing 1425 mg of protein

FIGURE 6

IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS OF TOXIN, TOXOID, AND NSP

a. Immunodiffusion

A. IMMUNODIFFUSION

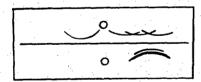| I	II	III
1-Toxin 5 mg/ml	1-Antitoxin 30 mg/ml	1-Antitoxin 30 mg/ml
2-NSP 16-3a 5 mg/ml	2-Antitoxin 15 mg/ml	2-Antitoxin 15 mg/ml
3-Toxin 1 mg/ml	3-Antitoxin 10 mg/ml	3-Antitoxin 10 mg/ml
4-Toxoid 5 mg/ml	4-Antitoxin 5 mg/ml	4-Antitoxin 5 mg/ml
Antitoxin 30 mg/ml	Toxin 5 mg/ml	NSP 16-3a 5 mg/ml

B. IMMUNOELECTROPHORESIS

IV	V	VI
Top-Toxin 5 mg/ml	Top-Toxin 5 mg/ml	Top-NSP 16-3a 5 mg/ml
Bottom-Toxoid	Bottom-NSP 16-3a	Bottom-Toxoid 15 mg/ml
15 mg/ml	5 mg/ml	Trough-Antitoxin
Trough-Antitoxin	Trough-Antitoxin	30 mg/ml
30 mg/ml	30 mg/ml	

Comment: Figure I shows that one antigen common to toxoid and NSP is removed by CGC treatment

Figure II-heaviest line shows fastest diffusion

Figure III-evidence that 5th line, common to toxin and toxoid is absent in NSP

FIGURE 7

VARIATION OF α-OPTIMUM OF TOXOID WITH ANTITOXIN CONCENTRATION

FIGURE 8

VARIATION OF PRECIPITATE COMPOSITION AT
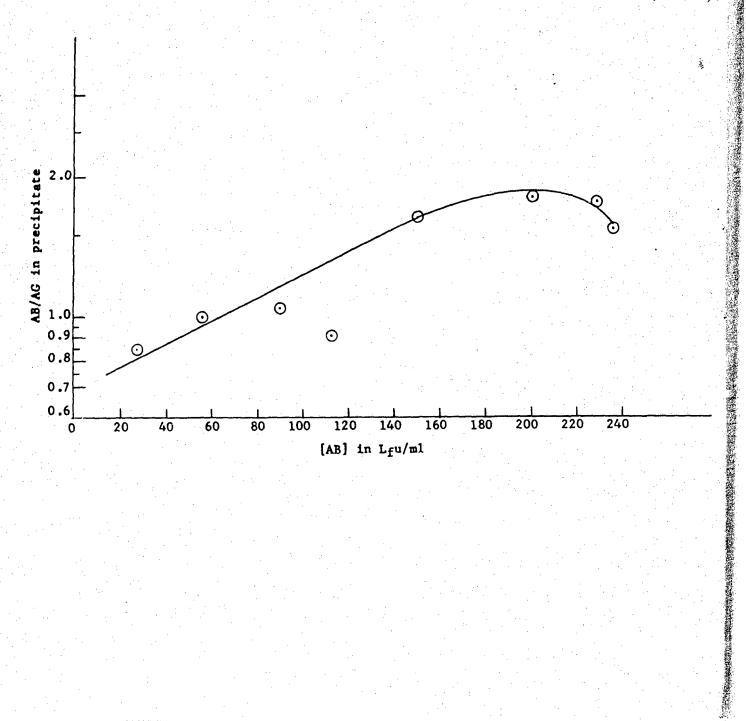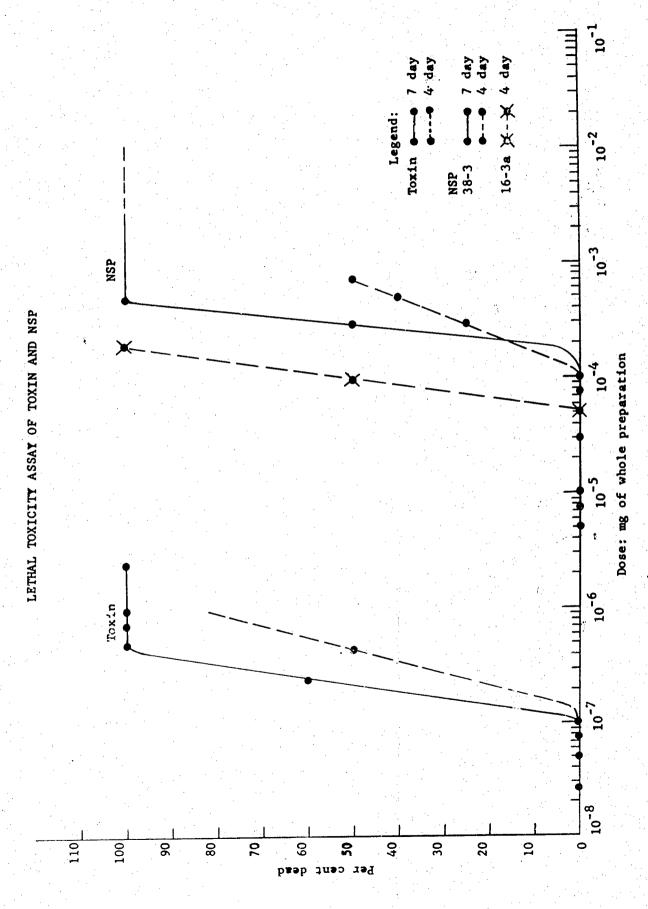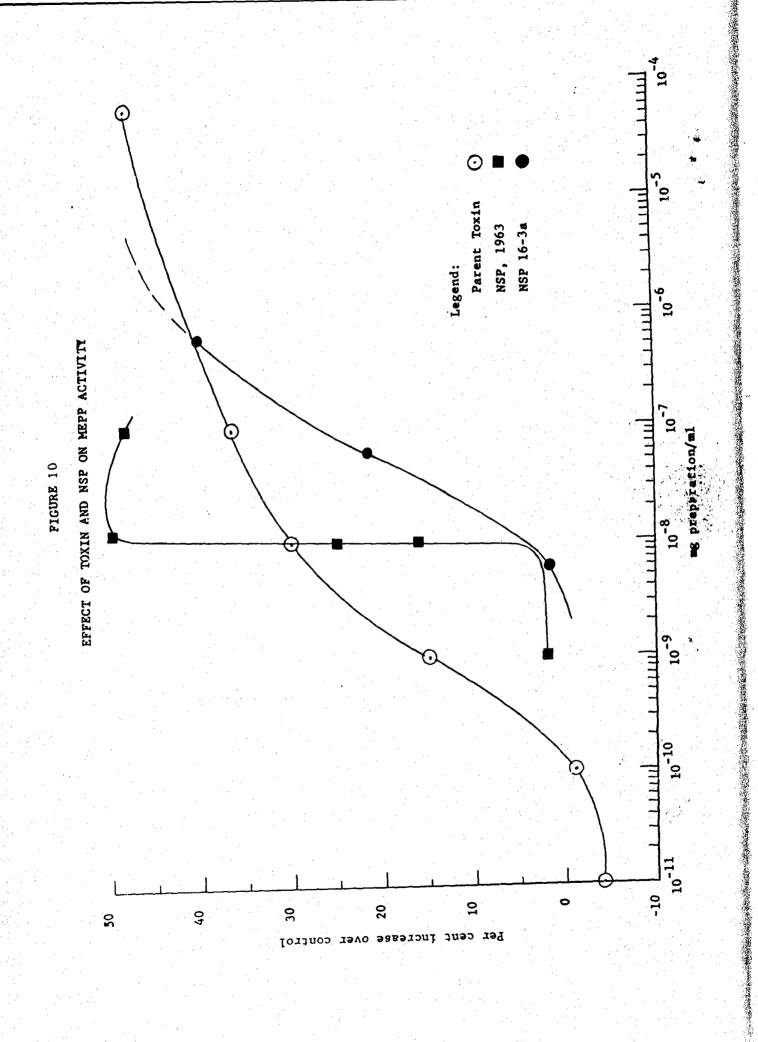EQUIVALENCE POINT WITH INCREASING ANTITOXIN CONCENTRATION

FIGURE 9

LETHAL TOXICITY ASSAY OF TOXIN AND NSP

FIGURE 10

EFFECT OF TOXIN AND NSP ON MEPP ACTIVITY

Legend:

⊙ Parent Toxin

■ NSP, 1963

● NSP 16-3a

PART III

PHARMACOLOGICAL BEHAVIOR OF SEA URCHIN TOXIN

III. PHARMACOLOGICAL BEHAVIOR OF SEA URCHIN TOXIN

The pedicelleriae of the Pacific sea urchin contain a protein toxin which can be isolated in relatively pure form by precipitation with ammonium sulfate. The product is a heat-labile material having the characteristics of an albumin according to solubility in ammonium sulfate.

A. Lethal Toxicity:

Dose-mortality studies were carried out on 33 mice, which were given intravenous doses ranging from 2.5 to 25γ per mouse in 0.10 ml of 1% NaCl solution. The per cent mortality at various dosage levels is exhibited in Table I. The LD_{50} was found to be $4.3\gamma/20g$ mouse. All deaths occurred either immediately or within 5 minutes of the injection; the survivors were alive 48 hours after the test and were discarded at that time.

B. Effects on Isolated Tissues of the Guinea Pig:

1. Gut: Sea urchin toxin causes guinea pig gut to contract. The dose-response characteristics of the system were studied according to the standard methods used in this laboratory (1,2) for concentrations of toxin varying from 4.5×10^{-4} to 2.85×10^{-2} mg/ml. The results are displayed in Table II and Figure 1. It is evident from Figure 1 that the dose-response curve is of the usual type and that the median effective dose is about 4.2×10^{-3} mg/ml as obtained graphically from a logistic plot of the results (Figure 1a from data in Table II).

The material released into the surrounding bath fluid following incubation at 37°C with 4.99×10^{-3} mg/ml SUT was found to contain both histamine and serotonin using the chemical assay methods of Shore, Burkhalter, and Cohen (3) for histamine and of Weissbach, Waalkes, and Udenfriend (4) for serotonin. These data are discussed in Section C below.

2. Heart: The perfusion of an effective dose of sea urchin toxin through the coronary circulation of the perfused guinea pig heart produces a progressive reduction in rate and amplitude and heart block. Associated with these changes in cardiac physiology is the progressively increasing quantity of histamine released as determined by the chemical assay method of Shore, et al (3). The results of cognizant experiments made with doses ranging from 4.96×10^{-5} to 6.24×10^{-4} mg sea urchin toxin/ml, are exhibited in Table III and displayed graphically in Figure 2. From Table III it appears that the maximal histamine output in response to sea urchin toxin is about 3×10^{-6} moles/g wet heart and that the concentration of sea urchin toxin giving a median response is about 1×10^{-4} mg/ml.

C. Kinetics of Humoral Release:

The time-course of humoral release at 37°C was studied by incubating 4 cm portions of guinea pig gut in aërated baths containing 4.99×10^{-3} mg of sea urchin toxin/ml and 1γ/ml semicarbazide. Samples were incubated for 15, 30, and 60 minutes. Following incubation a 10 ml portion of each perfusate was dialyzed against 50 ml of 10% NaCl for 48 hours to remove SUT and bioassayed on fresh gut preparations. The remainders of the perfusates were chemically assayed for histamine and serotonin as discussed in Section B1. The results given in Table IV show a progressive increase of both histamine and serotonin released over a 45 minute period.

D. Effects of Blocking Agents:

The action of sea urchin toxin on the gut was compared to the effects obtained with standard concentrations of histamine, serotonin, and acetylcholine. Pyribenzamine was used to block the response of the gut to histamine, atropine was employed to block that of acetylcholine, and d-brom-lysergic acid to block that of serotonin. Table Va shows that the reactions of the gut were blocked selectively by the appropriate blocking agents. The action of sea urchin toxin. however. as can be seen from Table Vb, was not blocked by either the histamine antagonist, pyribenzamine, or by atropine, and only partially by d-brom-lysergic acid, the serotonin antagonist.

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TABLE I

LETHAL TOXICITY OF SEA URCHIN TOXIN

SEA URCHIN TOXIN DOSE (γ/mouse)	NO. MICE	DIED	% MORTALITY
0.25	5	1/5	20
0.4	5	2/5	40
0.5	11	10/11	91
1.0	5	5/5	100
2.5	7	7/7	100

TABLE II

DOSE-RESPONSE RELATIONSHIP OF SEA URCHIN TOXIN ON GUINEA PIG GUT

NO. TISSUES	DOSE SEA URCHIN TOXIN: mg/ml	RESPONSE AS "HISTAMINE" EQUIVALENT moles/liter x 10^9	y	y/1-y
4	4.50×10^{-4}	$2.5 \pm 2.5*$	0.00417	0.00419
4	9.50×10^{-4}	46 ± 2	0.0767	0.0831
4	1.50×10^{-3}	77 ± 7.5	0.1283	0.1472
3	1.98×10^{-3}	86 ± 23	0.1433	0.1673
6	2.25×10^{-3}	114 ± 3.5	0.1900	0.2346
3	4.00×10^{-3}	340 ± 12	0.5667	1.3078
2	8.20×10^{-3}	430 ± 25	0.7167	2.5298
2	2.85×10^{-2}	600 ± 35	1.0000	∞

* Standard error of the mean

TABLE III

PERFUSION OF GUINEA PIG HEART WITH SEA URCHIN TOXIN

CHALLENGE Dose of SUT (mg/ml)	NO. HEARTS	WET HEART WEIGHT (gm)	FLOW $(ml \times g^{-1}$ wet wt $\times$ min$^{-1})$	HISTAMINE RELEASE (moles $\times$ liter^{-1} $\times$ g^{-1} wet weight)
0 (control)	23	1.85 ± 0.55	3.41 ± 0.84	0
4.96×10^{-5}	4	2.30 ± 0.31	0.377 ± 0.10	$(.50 \pm .10) \times 10^{-6}$
8.96×10^{-5}	4	1.84 ± 0.14	0.293 ± 0.078	$(1.13 \pm .23) \times 10^{-6}$
9.90×10^{-5}	3	1.42 ± 0.12	0.675 ± 0.047	$(1.40 \pm .16) \times 10^{-6}$
1.25×10^{-4}	3	1.21 ± 0.09	0.554 ± 0.11	$(2.11) \times 10^{-6}$
1.99×10^{-4}	3	2.31 ± 0.02	0.806 ± 0.036	$(2.50 \pm .10) \times 10^{-6}$
4.95×10^{-4}	2	2.40 ± 0.10	0.345 ± 0.017	$(3.00 \pm .05) \times 10^{-6}$
6.24×10^{-4}	4	1.46 ± 0.20	0.108 ± 0.09	$(2.91 \pm .24) \times 10^{-6}$

* Standard error of the mean

TABLE IV

TIME-COURSE OF HUMORAL RELEASE FROM THE GUINEA PIG GUT IN

SEA URCHIN TOXIN (4.99×10^{-3} mg/ml at 37°C)

NO. GUTS	INCUBATION TIME minutes	EQUIVALENT "HISTAMINE" RELEASE (determined by bio-assay) moles x liter^{-1} x mg^{-1} dry tissue	HISTAMINE RELEASE (determined by chemical assay) moles x liter^{-1} x mg^{-1} dry tissue	SEROTONIN RELEASE (determined by chemical assay) moles x liter^{-1} x mg^{-1} dry tissue
4	15	$(7.2 \pm 0.5) \times 10^{-9}$*	$(3.8 \pm 1.8) \times 10^{-9}$	$(1.4 \pm 0.5) \times 10^{-9}$
4	30	$(9.3 \pm 0.7) \times 10^{-9}$	$(5.4 \pm 1.0) \times 10^{-9}$	$(2.15 \pm 1.5) \times 10^{-9}$
4	60	$(1.22 \pm 1.0) \times 10^{-8}$	$(6.1 \pm 0.5) \times 10^{-9}$	$(2.62 \pm 2.0) \times 10^{-9}$

* Standard error of the mean

TABLE V-a

STUDIES OF BLOCKING AGENTS ON GUINEA PIG ILEUM:

Response (% of maximal) before and after blocking agents

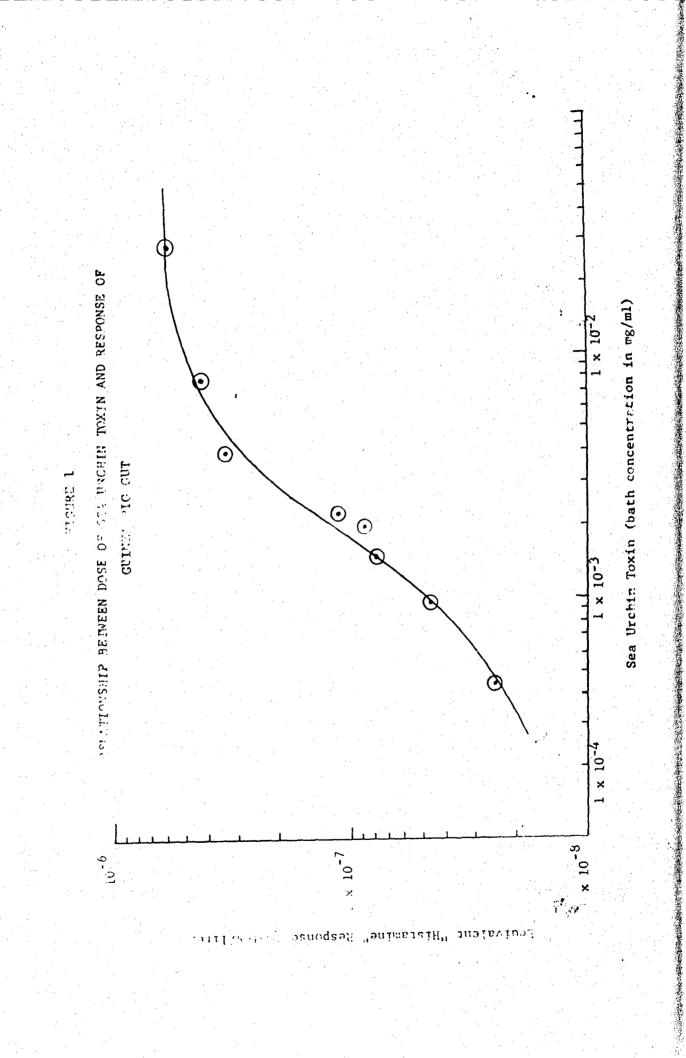| CONCENTRATION TEST SUBSTANCE	% RESPONSE BEFORE BLOCKING AGENT	% RESPONSE REMAINING AFTER BLOCKING AGENT		
		Pyribenzamine (0.05γ/ml)	Atropine (1.3γ/ml)	d-brom lysergic acid (0.005 mg/ml)
Histamine (moles/liter)				
1.66×10^{-8}	9 ± 2	0	5 ± 1	
6.00×10^{-8}	32 ± 3	0	32 ± 4	
6.60×10^{-8}	29 ± 1.5	0	25 ± 2	
8.00×10^{-8}	30 ± 2	0	32 ± 3	32 ± 5
1.00×10^{-7}	42 ± 3	0	33 ± 5	
1.33×10^{-7}	56 ± 1	0	38 ± 4	
5.00×10^{-7}	78 ± 3	1.5*	70 ± 5	
1.00×10^{-6}	100	2.5*	80 ± 6	
Acetylcholine (γ/ml)				
0.003	18 ± 3.0	19 ± 3	0	
0.025	60 ± 4.0	54 ± 2	0	
0.100	90 ± 3.5		11.5	
Serotonin (moles/liter)				
1.51×10^{-8}	21 ± 4.0			0
3.03×10^{-8}	31 ± 2.0			0
4.00×10^{-8}	37 ± 3.5			0
5.00×10^{-8}	46 ± 3.0			0
6.30×10^{-8}	50 ± 1.5			0
1.00×10^{-7}	58 ± 2.5			0

* Maximum response observed

TABLE V-b

EFFECT OF BLOCKING AGENTS ON RESPONSE OF
GUINEA PIG ILEUM TO SEA URCHIN TOXIN

CONCENTRATION SEA URCHIN TOXIN (mg/ml)	% RESPONSE BEFORE BLOCKING AGENT	% RESPONSE REMAINING AFTER BLOCKING AGENT		
		Pyribenzamine	Atropine	d-brom lysergic acid
1.10×10^{-3}	40 ± 2.0	40 ± 5	33 ± 3.5	
1.50×10^{-3}	50 ± 1.5			$17 \pm 3*$
1.98×10^{-3}	62 ± 2.0			$30 \pm 5*$
2.33×10^{-3}	73 ± 3.0			$45 \pm 2*$
2.85×10^{-3}	81 ± 1.0		75 ± 2	
3.00×10^{-3}	92 ± 5.0	90 ± 2		

* These guts were initially challenged with sea urchin toxin, rechallenged with
all concentrations of serotonin (see Table V-a), rechallenged again with
serotonin after d-brom-lysergic acid (no response--Table V-a), then lastly
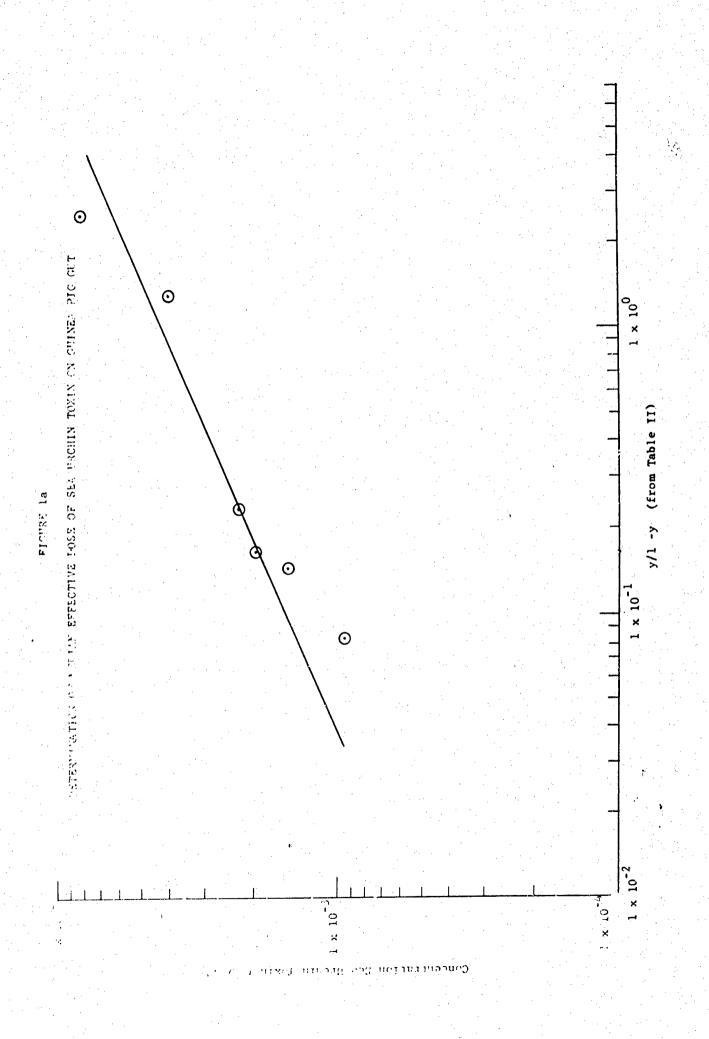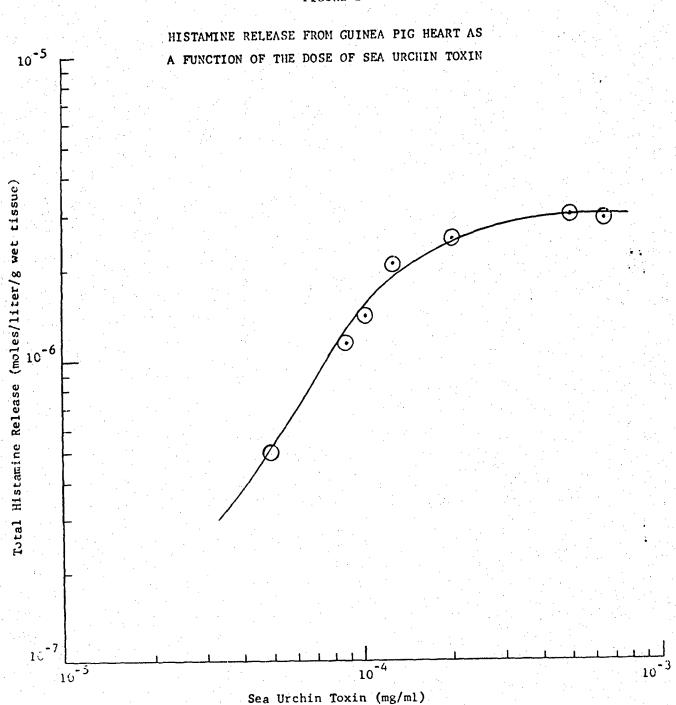rechallenged again with Sea Urchin Toxin.

FIGURE 1

RELATIONSHIP BETWEEN DOSE OF SEA URCHIN TOXIN AND RESPONSE OF
GUINEA PIG GUT

FIGURE 1a

DETERMINATION OF AN EFFECTIVE DOSE OF SEA URCHIN TOXIN ON GUINEA PIG GUT

Concentration Sea Urchin Toxin (g/ml)

$y/1 - y$ (from Table II)

1×10^{-2} 1×10^{-1} 1×10^{0}

FIGURE 2

HISTAMINE RELEASE FROM GUINEA PIG HEART AS
A FUNCTION OF THE DOSE OF SEA URCHIN TOXIN

PART IV

THE EFFECT OF STREPTOLYSIN ON THE RELEASE OF HUMORAL AGENTS FROM GUINEA PIG
TISSUES IN RELATION TO THE ELECTROLYTE CONTENT OF THE HEART

IV. THE EFFECT OF STREPTOLYSIN ON THE RELEASE OF HUMORAL AGENTS FROM
GUINEA PIG TISSUES IN RELATION TO THE ELECTROLYTE CONTENT OF THE
HEART

Halbert and coworkers (1) have demonstrated that the administration
of Group C streptolysin "O" produced cardiac irregularities in rabbits
which had received an effective dose of the toxin. Since the changes in
the electrocardiographic patterns in the test animals were very similar to
those seen in rabbits receiving high doses of potassium, they inferred that
streptolysin acted by releasing large, presumably fatal, quantities of
potassium from the tissues. These workers found, in subsequent experiments
that these physiological findings could be prevented by the administration
of serotonin blocking agents.

Our studies were based on the inference that the toxin releases a
humoral agent--histamine or serotonin--and that the EKG changes observed
are a reflection of electrolyte shifts which occur in consequence of the
release of humoral materials. The experiments reported in this communica-
tion have been made to test this hypothesis.

A. Methods

1. Streptolysin: Several batches of dry streptolysin "O" of the
Group C strain were obtained through the courtesy of Professor S. P. Halbert,
prepared according to the methods of his laboratory (1). Activation of the
streptolysin "O" solutions was carried out with 1-cysteine-HCl (1000 mg %)
in 0.15 M phosphate buffer at pH 7.2 as described by Halbert (2). Samples
remaining after a day's experimentation were discarded.

2. Biological Tests: Lethal toxicity was determined by injecting
mice intravenously with 0.1 ml doses of various concentrations of the strep-
tolysin. Isolated organ tests were conducted on perfused pig hearts (3) and
histamine and serotonin were estimated either chemically or biologically (4).

3. Water and Electrolytes: The procedures for the estimation of
inulin space and for the analyses for Na, K, Cl are those discussed in a recent
publication from this laboratory (5).

B. Results

1. Lethal Toxicity: Eighty Swiss-Webster mice were intravenously
injected with 0.1 ml doses containing 1.9 to 29 μg of crystalline toxin.
From the dose-mortality function, shown in Figure 1, the LD_{50} was found to
be about 13 μg/20g mouse.

2. Heart Perfusion: Guinea pig hearts were prepared and perfused
with Chenoweth's solution at 37°C solution according to the usual procedure
in this laboratory (3). In order to prevent excessive destruction of histamine
by tissue histaminases, a sufficient quantity of semicarbazide, 1γ/ml, was
added to the perfusing media. After an equilibration period of 20 minutes
in duration, streptolysin was added to the medium in doses ranging from
3.3×10^{-5} to 6.6×10^{-3} mg/ml. Effective doses of toxin produced first
an A-V block and then an irreversible arrest of the isolated heart.

3. <u>Serotonin and Histamine Output</u>: Pilot experiments showed that the heart regularly released serotonin but only occasionally released histamine in response to an effective dose of streptolysin. As can be seen from Figure 2 the output of serotonin ranged from 5×10^{-10} to 5×10^{-7} moles/gm wet heart for streptolysin doses varying between 3.3×10^{-5} and 6.7×10^{-3} mg/ml.

C. <u>Water and Electrolytes</u>

1. <u>Water</u>: The total water content of the cardiac tissues was essentially unaltered by treatment with streptolysin

2. <u>Na, K, and Cl</u>: Perfused hearts treated for no more than 5 minutes with 5.5×10^{-3} mg/ml streptolysin "O" after a 20 minute equilibration period in Chenoweth's solution were analyzed for Na, K, and Cl. As can be seen from Table I, total Na and total Cl increased while total K decreased following treatment with streptolysin. Since inulin space determinations have not yet been complete, compartmental shifts cannot be calculated, but a general appraisal of the results of streptolysin treatment can be got from the statistical information presented in Table II.

Briefly, the ionic patterns displayed by cardiac tissues treated with streptolysin are those consistent with the gross electrocardiographic findings of Halbert and coworkers of a loss of tissue K as the consequence of treatment. The tissue K lost appears to be reflected by an increase of K appearing in the perfusate; the expected exchange of Na for K is observed in the tissues analytically and is consistent in magnitude, within experimental error. Thus the ventricles which showed a greater loss in K content than the atria (-21.6% as against -10.4%) have a corresponding greater gain in tissue Na (+8.6% vs + 2.5%) than the latter. These data suggest that the consequence of streptolysin treatment may be to increase ionic permeability selectively and thus to alter the ionic composition according to the restrictions imposed on diffusible ions by the Donnan equilibrium. Since experiments of the direct effects of serotonin have not yet been made, it is impossible to state whether these changes are owed to the direct effects of the toxin or whether they are attributable to the release of serotonin which has been demonstrated to occur.

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TABLE I

ION AND WATER BALANCES IN STREPTOLYSIN-TREATED HEARTS

	CONTROLS			STREPTOLYSIN		
	Atria	Ventricles	Perfusate	Atria	Ventricles	Perfusate
H_2O Content (% of Wet Wt)	80.9 ± 0.3%* (10)	82.8 ± 0.3% (10)		81.3 ± 0.2% (10)	83.2 ± 0.3% (8)	
Inulin Space (% of Total H_2O)	27.7 ± 0.2% (8)	31.1 ± 2.9% (8)		work in progress		
Cl^- Content (mEq/100g dry wt)	31.9 ± 0.8 (10)	35.3 ± 1.1 (10)	110.2 ± 1.6 (10)	36.1 ± 1.0 (10)	38.9 ± 0.9 (10)	112.2 ± 1.6 (10)
K^+ Content (mEq/100g dry wt)	37.6 ± 0.7 (10)	43.0 ± 0.6 (9)	5.8 ± 0.2 (3)	33.7 ± 0.6 (10)	33.7 ± 0.4 (10)	6.6 ± 0.2 (3)
Na^+ Content (mEq/100g dry wt)	35.7 ± 0.9 (10)	35.0 ± 1.1 (9)	115.3 ± 1.2 (5)	36.6 ± 1.1 (9)	38.0 ± 1.1 (10)	116.9 ± 2.8 (5)

* Figures given are average values and standard errors for the number of tissue samples, n, indicated in parentheses. The number of hearts represented in each instance is n/2.
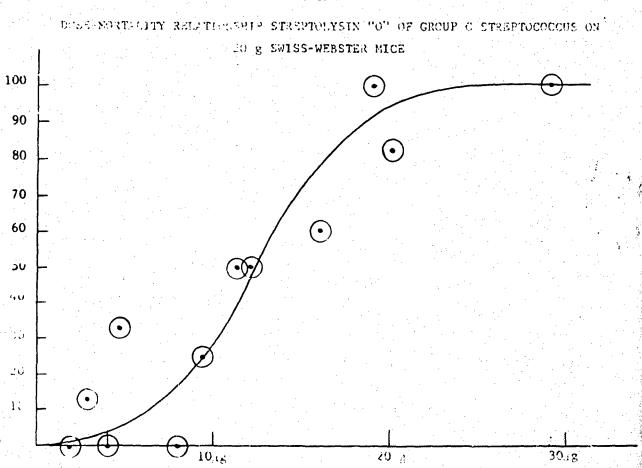
TABLE II

STATISTICAL ANALYSIS OF WATER AND ELECTROLYTE CHANGES

	% CHANGE IN STREPTOLYSIN HEARTS			P*		
	ATRIA	VENTRICLES	PERFUSATE	ATRIA	VENTRICLES	PERFUSATE
H_2O Content	+ 0.5%	+ 0.57%	--	0.3	0.4	--
Inulin Space	--	--	--	--	--	--
Cl^- Content	+13.27%	+10.2	+ 1.87	< .01	.02	0.4
K^+ Content	-10.4	-21.6	+13.8	< .01	< .01	0.05
Na^+ Content	+ 2.5	+ 8.6	+ 1.4	0.5	0.05	0.6

* As determined by student's T-test

FIGURE 1

DOSE-MORTALITY RELATIONSHIP STREPTOLYSIN "O" OF GROUP C STREPTOCOCCUS ON 20 g SWISS-WEBSTER MICE
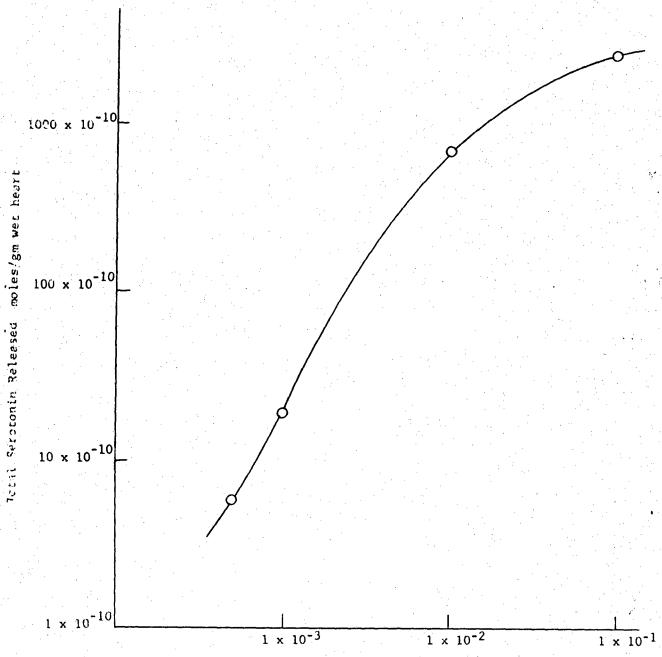
FIGURE 2

DOSE-RESPONSE RELATION OF SEROTONIN TO STREPTOLYSIN "O" FROM GUINEA PIG HEARTS, PERFUSED WITH CHENOWETH'S SOLUTION.